Received: 10 July 2024 | Accepted: 29 October 2024

DOI: 10.1002/fpf2.12039

REVIEW

WILEY

Guideline for anticancer assays in cells

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Abstract

Evaluation of anticancer effects on cell levels plays a crucial role in drug discovery. This guideline summarizes various methods for assessing anticancer effects and mechanisms, including cell viability, cell cycle, cell metabolism, cell damage and death, cell behaviors and function, it also briefly reviews the basic principles and specific experimental procedures. It is worth noting that it is recommended to use more than three methods to verify the accuracy of experimental results, so as to provide necessary references for the screening and evaluation of anticancer drugs and foods.

KEYWORDS
anticancer, cell apoptosis, cell viability, guideline, molecular mechanism

1 | INTRODUCTION

Cancer, as a major global public health issue, poses a severe threat to human well-being. In 2018, the World Health Organization reported that cancer is the second leading cause of death worldwide, resulting in approximately 9.6 million deaths annually (Ferlay et al., 2019). Traditional cancer treatments often involve inducing cell death, such as radiotherapy and chemotherapy (Gupta et al., 2022; Makker et al., 2021). Some chemotherapeutic drugs, such as doxorubicin and cisplatin, are commonly used to treat most cancers, but they come with severe adverse reactions and toxic side effects. In recent years, natural compounds have played a key role in cancer prevention and treatment due to their lower toxicity and multi-target anticancer activities. Natural ingredients such as curcumin, quercetin, icariin, berberine, and piperine can promote apoptosis and prevent invasion and metastasis, thereby exerting significant anticancer effects. Selecting appropriate methods to detect apoptosis, invasion, and metastasis is crucial during the drug screening process (Dehelean et al., 2023).

In cancer research, measuring cell viability is a crucial method for evaluating treatment efficacy. Numerous studies have preliminarily assessed the anticancer effects of drugs in vitro by measuring changes in cell viability levels following drug treatment. Since the process of cell death typically involves alterations in various physiological functions and target pathways within cells, multiple methods are often combined to reveal the causes of drug-induced cell death (Chen et al., 2023). These methods include cell cycle analysis, mitochondrial membrane potential (MMP) assessment, DNA damage detection, Ca^{2+} and reactive oxygen species (ROS) level testing, and the measurement of apoptosis, migration, and invasion. Additionally, the Transwell assay was employed to measure the effects on cell migration and invasion. Building on these findings, western blotting (WB) and immunofluorescence (IF) techniques were used to validate the molecular-level targeting of signaling pathways, thereby elucidating the underlying mechanisms (Tang et al., 2023; Zhang, Zheng, et al., 2023).

In the study of anticancer activity, an increasing number of researchers are focusing on in vitro analysis of cellular drug testing. Therefore, this guide summarizes the common detection methods used in the screening process of anticancer drugs or foods. It reviews the basic principles, reagents, instruments, and specific experimental procedures associated with these methods, aiming to provide stronger support for research on anticancer drugs or foods.

2 | SELECTION OF CELL MODELS

Different types of cancer are typically associated with specific cancer cell lines. Therefore, in research, selecting the appropriate cancer cell line based on the characteristics of the cancer type is crucial. The American Type Culture Collection, one of the world's largest cell banks (https://www.atcc.org), provides a wide variety of cancer cell lines, primary cells, and detailed descriptions of cell lines, including culture conditions and background information (Mirabelli et al., 2019). Generally, multiple cancer cell lines correspond to a single type of cancer, allowing for selection based on the specific characteristics of the cell lines in actual research.

3 | CELL CULTURE

Cell culture is a necessary prerequisite for conducting anticancer experiments. Due to the differences in culture conditions and growth cycles among various cancer cell lines, it is essential to select and adjust according to the specific circumstances.

3.1 | Overview of culture systems

Based on the type of culture systems, cancer cells can be categorized into suspension growth, semi-suspension growth, and adherent growth types. Suspension growth cancer cells remain in a suspended state during culture, not adhering to the surface of the culture dish. Examples include certain hematological malignancies such as leukemia or lymphoma cells. Semi-suspension growth cancer cells have some cells adhering to the surface of the culture dish while others remain suspended in the culture medium. Examples include U937 and K562 cells. This type typically requires special culture conditions to maintain the cell growth state. Adherent growth cancer cells require attachment to the surface of the culture dish for growth, relying on interactions between the cells and the substrate. The majority of tumor cell lines belong to this category, including many common solid tumor-derived cell lines such as breast cancer cells, lung cancer cells, and colorectal cancer cells. There are some differences in how these three types of cancer cells are cultured in vitro (Mirabelli et al., 2019).

3.2 | Culture methods

When the cell density reaches approximately 80%, discard the culture medium in a sterile hood after UV sterilization. Gently wash the cell surface with phophate buffered saline (PBS, pH 7.4) to remove any residual culture medium. Add an appropriate amount of trypsin (0.05%–0.25%) and incubate at 37°C for 3–5 min. Adjust the digestion time based on the actual digestion effect, ensuring cells detach from the culture dish as observed under a microscope. Immediately add an equal volume of complete culture medium containing serum to stop the digestion process. Collect the digested cells into a centrifuge tube using a pipette. Centrifuge at 1000 g for 3–5 min, discard the supernatant, and resuspend the cell pellet in fresh culture medium. Count the cells using a cell counter or a hemocytometer. Transfer the cells at an appropriate ratio into new culture medium and continue incubation at 37°C with 5% CO_2. Regularly observe the cell growth and perform subculturing or experiments as needed. For suspension cells, collect the cells from the culture medium by centrifugation, then add fresh culture medium. For semi-suspension cells, collect both the cells suspended in the medium and those adhering to the dish by using trypsin to digest and detach the adherent cells (Ma et al., 2020).

4 | DETERMINATION OF CELL VIABILITY

There are various methods for determining cell viability, with the most common being 3- (4,5-Dimethylthiazol-2-yl) -2,5-diphenyltetrazoliumbromide (MTT), CCK-8, lactatedehydrogenase (LDH), Trypan blue exclusion assay, and adenosine triphosphate (ATP) assay (Adan et al., 2016; Kamiloglu et al., 2020). The MTT assay is one of the most widely used methods for determining cell viability due to its simplicity, low cost, and suitability for high-throughput screening. However, it cannot dynamically monitor changes in cell viability. On the other hand, the CCK-8 and LDH assays can continuously monitor cell viability changes but have higher reagent costs and stringent detection conditions. Additionally, the trypan blue exclusion assay requires manual counting, which is usually time-consuming and prone to errors. The ATP assay is highly sensitive but prone to errors due to the rapid degradation of ATP, and its high cost makes it more suitable for assessing cellular metabolic activity (Table 1, Lu et al., 2012; Strober, 2015). Therefore, there are many methods available for determining cell viability, each with its own advantages and limitations. The choice of method should be based on the specific experimental conditions. The following sections will focus on detailed guidelines for MTT and CCK-8 assays.

4.1 | MTT assay

4.1.1 | Principle

The MTT assay, also known as the MTT colorimetric assay, is based on the ability of mitochondrial succinate dehydrogenase in living cells to reduce exogenous MTT to insoluble blue-purple formazan crystals. Only metabolically active cells can convert MTT into formazan, while dead cells lack this capability. The intensity of the colored product is proportional to the number of viable cells in the culture (Figure 1). Therefore, the MTT assay is one of the most commonly used methods for detecting cell viability and proliferation (Ghasemi et al., 2021).

4.1.2 | Experimental procedure

Collect the cells in the logarithmic growth phase, count them under the microscope with a cell counting plate, add culture medium to resuspend the cells to reach a suitable concentration, take 100 μL of

TABLE 1 Summary of methods for assessing cell viability.

Method	Principle	Advantages	Disadvantages	Application
MTT assay	MTT is reduced by mitochondrial dehydrogenases to form purple formazan crystals, measured by absorbance at 570 nm	Simple operation, low cost, and suitable for high-throughput screening	Requires dissolving formazan crystals, multiple steps involved	Drug screening, toxicity testing, and cell growth studies
CCK-8 assay	WST-8 is reduced by dehydrogenases to form water-soluble formazan, measured by absorbance at 450 nm	Easy to use, high sensitivity, no dissolution step required, and suitable for continuous measurement	Higher reagent cost	Drug screening and cell proliferation assays
LDH assay	Measures lactate dehydrogenase released into the medium, indicating cell damage or death	Nondestructive and suitable for dynamic monitoring	Requires stringent detection conditions, LDH stability is limited	Cytotoxicity testing, cell damage studies
Trypan blue exclusion assay	Trypan blue dye detects membrane integrity; viable cells exclude the dye while dead cells are stained	Simple operation and low cost	Manual counting required, time-consuming, and prone to errors	Cell viability assessment and cell culture
ATP assay	Measures intracellular ATP levels to reflect metabolic activity	High sensitivity and rapid results	ATP is unstable and samples must be handled carefully, higher cost	Metabolic activity evaluation and drug screening

Abbreviations: ATP, adenosine triphosphate; LDH, lactatedehydrogenase; MTT, 3- (4,5-Dimethylthiazol-2-yl) -2,5-diphenyltetrazoliumbromide.

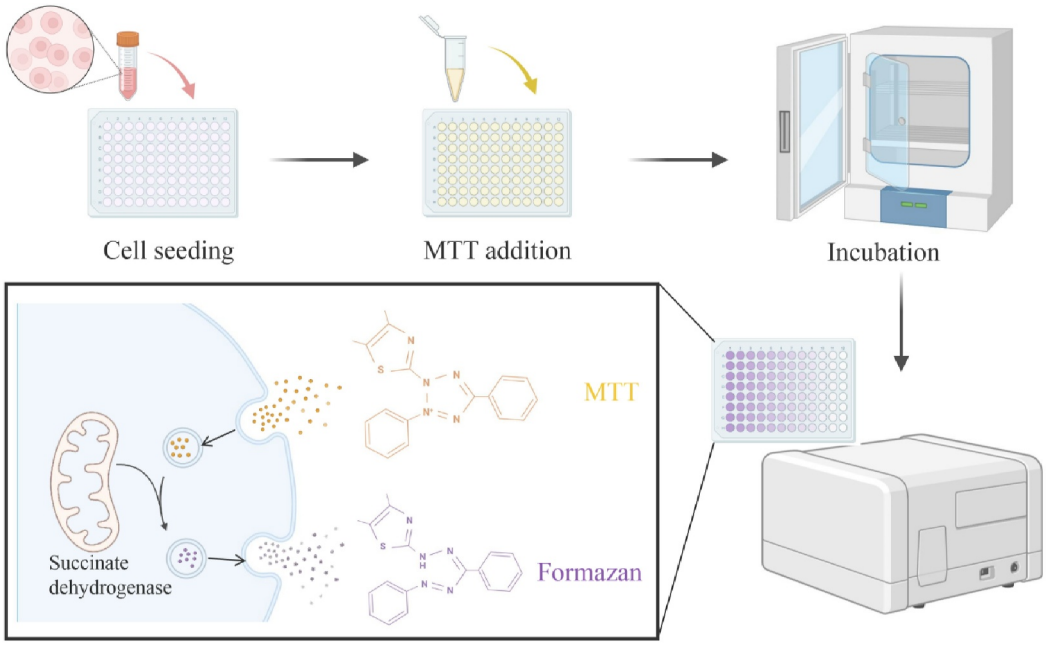

FIGURE 1 Diagram of the experimental procedure for MTT. MTT, 3- (4,5-Dimethylthiazol-2-yl) -2,5-diphenyltetrazoliumbromide.

cells and add them into a 96-well plate, and put it in an incubator to make the cells fully adhered to the wall. After drug action, aspirate the culture medium from the 96-well plate and wash gently with PBS (to prevent the drug from reacting with the MTT and affecting the accuracy of the results), then add 100 μL of cell culture medium and 20 μL of MTT at a concentration of 5 mg/ml to each well. The MTT is dissolved in sterile PBS and filtered through a 0.2 μm filter. The 96-well plate was gently shaken to mix well, and then incubated at 37°C for 4 h in an incubator protected from light. 150 μL of dimethyl sulfoxide (DMSO) was added to each well, and then placed on a shaker and shook for 10 min at room temperature, protected from light, so as to make mezanine dissolve completely, and then quickly detected the absorbance at 570 nm by an enzyme counter. DMSO was used as a negative control and the inhibition of cell proliferation by the drugs was calculated according to the formula for calculating the IC_{50} value (concentration of drug corresponding to 50% inhibition of cell proliferation) by nonlinear fitting of the Boltzman model for cell growth using Origin software (Li et al., 2021).

$$\text{Cell proliferation inhibition rate } (\%) = \left(OD_{\text{control group}} - OD_{\text{drug-treated group}}\right) / \left(OD_{\text{control group}} - OD_{\text{blank group}}\right) \times 100\%$$

4.2 | CCK8 assay

4.2.1 | Principle

The principle of the CCK-8 method is similar to MTT assay. The WST-8 reagent is converted by mitochondrial dehydrogenases in viable cells to form water-soluble formazan. The amount of formazan produced is proportional to the number of cells. The optical density (OD) of the cell culture medium is measured at 450 nm using a microplate reader, which indirectly reflects the number of viable cells.

4.2.2 | Experimental procedure

According to experimental needs, cells were seeded into a 96-well plate, adding an appropriate amount of cell suspension to each well (usually 100 μL). The cell number should be optimized based on cell type and experimental purpose, typically ranging from 20,000–100,000 cells per well. Incubate the cell culture plate at 37°C with 5% CO_2 until the cells adhere and reach an appropriate growth state. Add the drug solution to the corresponding wells, setting different concentration gradients, and continue to incubate in the incubator for an appropriate time (usually 24–48 h). Remove the 96-well plate from the incubator and add 10 μL of CCK-8 reagent to each well, being careful to avoid bubble formation. Gently shake the plate to ensure the CCK-8 reagent is evenly distributed. Return the 96-well plate to the incubator at 37°C with 5% CO_2 and incubate for 1–4 h (the specific time should be determined based on cell type and experimental conditions). After incubation, remove the 96-well plate from the incubator. Measure the absorbance (OD value) of each well at 450 nm using a microplate reader (Wang et al., 2023). Use wells containing only culture medium without cells as blank controls. Calculate the relative cell proliferation rate or viability for each well.

$$\text{Cell growth inhibition rate } (\%) = \left(\text{OD}_{\text{control}} - \text{OD}_{\text{experimental}}\right)/\text{OD}_{\text{control}} \times 100\%.$$

5 | CELL CYCLE ANALYSIS

5.1 | Principle

The cell cycle consists of distinct phases: G0/G1 phase, S phase, and G2/M phase. Each phase is characterized by different DNA content. Propidium iodide (PI), a DNA dye that binds to double-stranded DNA and fluoresces, is commonly used to stain DNA. The fluorescence intensity of PI is proportional to the amount of DNA present (Zaher et al., 2016). The flow cytometer uses a 488 nm laser to excite a PI fluorescent dye in the cells and detects the intensity of the emitted 617 nm fluorescence. As the cells pass through the detection channel of the flow cytometer one by one, the fluorescence intensity is recorded, and a histogram of the fluorescence intensity is formed. Based on the fluorescence intensity (McKinnon, 2018), the DNA content of each cell can be determined, thus analyzing which cell cycle phase it is in.

5.2 | Experimental procedure

The treated cells were collected into a centrifuge tube and centrifuged at 1000 rpm for 5 min and the supernatant was discarded. The cells were resuspended with PBS and centrifuged again. After discarding the PBS, add precooled 70% ethanol to fix the cells, mix gently, and place at 4°C overnight. Centrifuge at 1000 g for 5 min, discard the ethanol, and wash the cells with PBS to remove excess ethanol. The cells were resuspended with appropriate amount of PBS, added with RNAase A, and incubated at 37°C for 30 min to remove RNA. Propidium iodide (50 μg/ml) was added for staining, and the cells were incubated for at least 30 min, protected from light. Subsequently, the cells were up-registered by flow cytometry. Appropriate excitation and detection wavelengths were set (excitation wavelength of 488 nm and emission wavelength of 617 nm for PI staining). Data are collected and analyzed, usually using specialized software FlowJo (Matthews et al., 2022).

6 | MEASUREMENT OF CELLULAR METABOLISM

6.1 | Mitochondrial Membrane Potential assay

6.1.1 | Principle

Mitochondrial membrane potential (Δψm) is an important indicator of mitochondrial function, reflecting the electrochemical gradient between the inner and outer membranes of the mitochondria. The maintenance of Δψm is essential for ATP synthesis and cellular energy metabolism. Changes in MMP are often associated with processes such as apoptosis and oxidative stress. The basic principle of Δψm assay is the use of fluorescent dyes (JC-1, tetramethylrhodamine methyl ester, tetramethylrhodamine ethyl ester perchlorate, etc.), which are capable of generating different fluorescent signals under different Δψm conditions (Wang, Xie, et al., 2022). Taking JC-1 as an example, JC-1 is a dual fluorescent dye. At high Δψm, JC-1 forms J-aggregates and emits red fluorescence ($\lambda_{em} = 590$ nm); at low Δψm, JC-1 exists as a monomer and emits green fluorescence ($\lambda_{em} = 530$ nm). The red/green fluorescence ratio can be used to quantify Δψm. A high red/green fluorescence ratio indicates a high MMP and normal mitochondrial function. A low red/green fluorescence ratio indicates a low MMP and possible mitochondrial dysfunction.

6.1.2 | Experimental procedure

JC-1 working solution (10 μg/ml) was prepared using DMSO, and 1 ml of the JC-1 working solution was add to each 1×10^6 cells/mL of cell suspension. Mix gently to ensure even distribution of the dye. Incubate the cells in a cell culture incubator at 37°C for 30 min to allow JC-1 dye to stain mitochondria, then the medium was carefully aspirated and the cells were washed slowly with precooled PBS 2–3 times to remove unbound dye. The stained cell suspension was quantified by flow cytometry. Appropriate excitation and emission wavelengths were set

to detect red fluorescence (λ_{ex} = 488 nm, λ_{em} = 590 nm) and green fluorescence (λ_{ex} = 488 nm, λ_{em} = 530 nm), respectively. Calculate the ratio of red fluorescence to green fluorescence to assess MMP (Δψm). For fluorescence imaging, prepare cells on glass slides after staining. Use a fluorescence microscope equipped with appropriate filters to observe and capture images of stained cells (Han et al., 2020).

6.2 | Measurement of intracellular Ca^{2+}

6.2.1 | Principle

Intracellular calcium ions (Ca^{2+}) serve as crucial signaling molecules in various biological processes including muscle contraction, neural transmission, cell proliferation, and apoptosis. Fluorescent dyes are commonly used to determine intracellular Ca^{2+} concentrations, such as Fura-2 acetoxymethyl (AM), Fluo-3 AM, Fluo-4 AM, and Rhod-2 AM, which specifically bind to Ca^{2+} and produce fluorescent signals (Deak et al., 2021). The principles, applications, and advantages/disadvantages of these three fluorescent dye methods are summarized in Table 2 (De et al., 2021; Jiang et al., 2023; Lee et al., 2021). Researchers typically select the appropriate fluorescent dye method based on experimental duration, speed of signal changes, requirements for signal intensity, and stability, among other factors. Below, using Fluo-4 AM as an example, specific experimental procedures are detailed.

6.2.2 | Experimental procedure

Prepare Fluo-4 working solution (0.5–5 μM) in a 6-well plate with 1 ml per well by diluting the Fluo-4 stock solution in serum-free culture medium or PBS. Add 1 ml of the Fluo-4 working solution to each well containing 1×10^6 cells/mL and mix gently. Incubate at 37°C in the dark for 30 min. After staining, carefully wash the cells 2–3 times with prechilled PBS to remove unbound dye. Subsequently, observe the staining effect under a fluorescence microscope. Flow cytometry can also be performed for detection ((Lee et al., 2021).

6.3 | Measurement of intracellular Reactive oxygen species

6.3.1 | Principle

Reactive oxygen species are a class of highly reactive molecules generated during cellular metabolism, including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH·), among others. The detection of ROS typically employs fluorescent probes, which upon reacting with ROS, produce fluorescence. This allows for indirect measurement of intracellular ROS levels based on changes in fluorescence intensity. Commonly used ROS probes include dichlorodihydrofluorescein diacetate (DCFH-DA) dihydroethidium (DHE), and MitoSOX Red. Among them, 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) is widely used for general ROS detection in various cell types due to its simplicity and high sensitivity. It is a nonfluorescent and nonpolar compound that freely penetrates cell membranes. Once inside cells, it is hydrolyzed by intracellular esterases to form DCFH, which is oxidized to fluorescent DCF in the presence of ROS. Dihydroethidium (DHE) primarily detects intracellular superoxide anion. It reacts with superoxide anion to generate fluorescent products. MitoSOX Red is specific for detecting mitochondrial superoxide anion production. It exhibits strong specificity for mitochondria. The experimental procedure of DCFH-DA will be described below as an example (Liang et al., 2021).

6.3.2 | Experimental procedure

DCFH-DA was diluted in serum-free culture medium to a final concentration of 10 μM. Cells (1×10^6 cells/mL) were collected and

TABLE 2 Summary of intracellular Ca^{2+} level fluorescence staining methods.

Fluorescent dye	Principle	Application	Advantages	Disadvantages
Fura-2	Dual-wavelength fluorescent probe, compares fluorescence ratios at 340 and 380 nm to indirectly measure Ca^{2+} concentration	Rapid dynamic changes in Ca^{2+} signals	High selectivity and sensitivity, suitable for dynamic Ca^{2+} measurement	Requires precise wavelength ratio calculation and calibration, relatively complex
Fluo-3/ Fluo-4	Single-wavelength fluorescent probe, emits green fluorescence upon Ca^{2+} binding, uses 488 nm excitation wavelength and 530 nm emission wavelength	Real-time monitoring of intracellular Ca^{2+} concentration changes	Simple operation, suitable for real-time monitoring of intracellular Ca^{2+} concentration changes	May be affected by cell staining efficiency and background fluorescence
Rhod-2	Emits red fluorescence upon Ca^{2+} binding, uses approximately 550 nm excitation wavelength and approximately 580 nm emission wavelength	Long-term monitoring of intracellular Ca^{2+} concentration changes	Suitable for long-term measurement, sensitive to long-term Ca^{2+} signal changes	Red fluorescence may be relatively weak in some cells or under certain conditions

suspended in the diluted DCFH-DA solution, then incubated in a cell culture incubator for 30 min. Every 3–5 min, the suspension was gently inverted to ensure thorough contact between the probe and cells. After washing the cells with serum-free culture medium, detection was performed with λ_{ex} at 488 nm and λ_{em} at 525 nm. Laser confocal microscopy, flow cytometry, and fluorescent enzyme markers can be used for up-sampling and testing (Ma et al., 2020).

7 | DETERMINATION OF CELL DAMAGE AND DEATH

7.1 | Comet assay

7.1.1 | Principle

The comet assay involves embedding individual cells in agarose gel, lysing them to release DNA from the nucleus, and subjecting them to an electric field. This process causes DNA fragments to migrate according to their damage levels, forming a comet-like structure. Undamaged DNA remains primarily in the nucleus (comet head), whereas damaged DNA forms the tail of the comet. By observing and quantifying the length and fluorescence intensity of the comet tail under a microscope, the extent of DNA damage can be assessed (Guedes et al., 2023; Muruzabal et al., 2021).

7.1.2 | Experimental procedure

Suspend cells in PBS and mix with low melting point agarose (0.5%–1%) while maintaining at 37°C in a water bath. Ensure that no air bubbles are formed during agarose preparation. Prepare 1% regular agarose and spread on glass slides, allowing it to solidify. Avoid bubble formation during this step. Mix an appropriate volume of cell suspension (1×10^6 cells/mL) with warm low melting point agarose and quickly drop it onto the preset regular agarose-coated slides. Cover with a coverslip and let it solidify. Transfer slides to a 10 cm petri dish, add 10 ml of precooled lysis solution to fully submerge slides, and incubate overnight at 4°C. Wash slides with PBS for 3 min. After equilibrating in precooled electrophoresis buffer for 1 h, place slides in an electrophoresis tank filled with electrophoresis buffer and run at 25 V for 30 min. After electrophoresis, remove slides and immerse in neutralization buffer 1–3 times for 5 min each, discard neutralization buffer. Apply approximately 20 μL of PI onto the slides, cover, and stain in the dark for 10–20 min. Wash the slides three times, cover with a coverslip, and observe under a fluorescence microscope (Figure 2).

7.2 | Staining detection of apoptosis

Apoptosis is a programmed cell death process that does not induce an inflammatory response in surrounding tissues. It plays a crucial role in maintaining tissue homeostasis, regulating developmental processes, and eliminating abnormal cells. Apoptotic cells typically exhibit distinct features such as cell shrinkage, nuclear condensation, DNA fragmentation, and changes in the cell membrane (Lakshmanan & Batra, 2013). Based on these characteristics, staining methods for detecting apoptosis include Annexin V/PI double staining, TUNEL staining, Hoechst 33342, and 4′,6-diamidino-2-phenylindole (DAPI) staining (Table 3). Each method has its own characteristics and scope of application. Each method has its own features and applicable scope. Annexin V/PI double staining can distinguish different stages of apoptotic cells and is simple to perform, but it cannot detect DNA breaks and fragmentation. TUNEL staining can directly detect DNA breaks and fragmentation with high accuracy, but the procedure is relatively complex. Hoechst 33342 or DAPI staining allows direct observation of the nuclear morphological changes in apoptotic cells, but it cannot directly detect DNA breaks and fragmentation. Additionally, these stains can also label live cells, making it difficult to distinguish between apoptotic and normal cells. In experiments, a combination of different apoptosis detection methods is often used to comprehensively assess the occurrence and extent of apoptosis, thereby improving the accuracy and reliability of apoptosis detection.

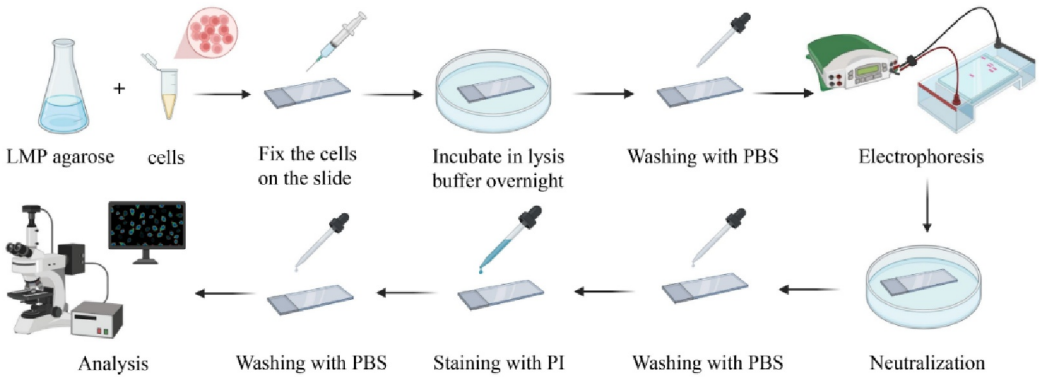

FIGURE 2 Illustration of the protocol for comet electrophoresis.

TABLE 3 Summary of methods for detecting apoptosis staining in cells.

Method	Principle	Application	Advantages	Disadvantages
Annexin V/PI double staining	Annexin V binds to externalized PS, PI stains to differentiate cell membrane integrity	Cell culture, cell suspensions	Differentiates early and late apoptosis, rapid and simple	Does not directly detect DNA fragmentation, requires differentiation of membrane integrity
Hoechst 33342 or DAPI staining	Labels cell nuclei DNA	Cell culture, tissue sections, cell suspensions	Quick and simple, direct observation of nuclear morphological changes	Does not directly detect DNA fragmentation, stains live cells, may require combination with other methods
TUNEL staining	Labels 3′OH ends of fragmented DNA with dUTP	Cell culture, tissue sections, cell suspensions	Direct detection of DNA fragmentation, high accuracy	Complex procedure, potential for background signals and time-consuming

Abbreviation: DAPI, 4′,6-diamidino-2-phenylindole.

7.2.1 | Annexin V/Propidium iodide double staining

Principle

In the early stage of apoptosis, phosphatidylserine (PS) turns over from the inside to the outside of the cell membrane. Annexin V can specifically bind to PS, by labeling Annexin V with the green fluorescent probe fluorescein Isothiocyanate (FITC), early apoptotic cells can be detected. Propidium iodide cannot penetrate the membrane of normal intact cells but can permeate late apoptotic or dead cells, staining the nucleus red. Therefore, using Annexin V in conjunction with PI allows for the distinction of cells at different stages of apoptosis based on fluorescence (Zhu et al., 2016).

Procedure

Cells to be tested were cultured in appropriate medium and collected directly after appropriate drug treatment. Wash the cells twice with precooled PBS, adjust the cell concentration to 1×10^6 cells/mL, and add 195 μL Annexin V-FITC conjugate to gently resuspend the cells. Add 5 μL Annexin V-FITC, mix gently, then add 10 μL PI staining solution, and then mix gently. Incubate for 10–20 min at room temperature under light-avoidance conditions, place the cells on ice after incubation. Immediately analyze the cells using a flow cytometer. Annexin V-FITC shows green fluorescence and PI shows red fluorescence. Fluorescence microscopy can also be used for detection, after staining, the cells were collected by centrifugation at 1000 g for 5 min, and the cells were gently resuspended with 50–100 μL of Annexin V-FITC conjugate, coated, and then observed and photographed under a fluorescence microscope.

7.2.2 | Hoechst 33258 staining

Principle

Hoechst 33342 is a fluorescent dye that can penetrate cell membranes and specifically bind to A-T base pairs in double-stranded DNA. After staining, it emits blue fluorescence ($\lambda_{em} \approx 461$ nm) when exposed to ultraviolet light ($\lambda_{ex} \approx 350$ nm). During apoptosis, the nuclei of apoptotic cells appear densely stained or fragmented with increased fluorescence intensity.

Procedure

Culture the cells in an appropriate medium and treat them with the relevant drugs. Collect the cells directly after treatment. Wash the cells twice with precooled PBS. If fixing the cells is necessary, use 4% paraformaldehyde to fix the cells for 10–15 min. Wash the fixed cells twice with PBS. Then, Add Hoechst 33258 staining solution (1–10 μg/ml) to cover the samples. Incubate at room temperature for 3–5 min. Wash the cells twice with PBS to remove unbound dye. Seal the film with an anti-fluorescence quenching sealer and place under a fluorescence microscope and photograph (Zheng et al., 2021).

7.2.3 | TUNEL staining

Principle

TUNEL (terminal deoxynucleotidyl transferase (TdT) deoxyuridine triphosphate (dUTP) Nick End Labeling) staining utilizes the deoxynucleotidyl transferase (TdT) to label the 3′ hydroxyl (3′OH) ends of DNA. Compared with normal cells, a large number of 3′ hydroxyl (3′OH) ends will be exposed in apoptotic cells. Through staining and observation, these labeled DNA fragments can be detected, thereby identifying the apoptotic cells. The labeled DNA fragments can then be detected through staining and observation to identify apoptotic cells (Rossello & Adell, 2023).

Experimental procedure

The treated cells were collected and fixed using 4% paraformaldehyde for 30 min, washed three times with PBS, and then permeabilized using 0.1% Triton X-100 for 10 min. After washing with PBS, cells were treated with TUNEL reaction solution for 1.5 h in a dark environment, then washed twice with PBS and incubated with DAPI (1 μg/ml) for 5 min. Stained cells were observed using a fluorescence microscope and images were taken to calculate the number and percentage of TUNEL-positive cells (Loo, 2011).

7.3 | Determining cell morphology

7.3.1 | Principle

Transmission electron microscopy is a method for detecting cell morphology and can clearly observe the fine internal structure of cells. It has extensive applications in the morphological detection of cell death. Apoptosis is usually accompanied by a decrease in cell volume, concentration of cytoplasm, and fragmentation of the nucleus to produce apoptotic bodies. During autophagy, autolysosomes, and autophagosomes are produced. In pyroptosis, cells form a large number of small vesicles, namely pyroptotic bodies. In ferroptosis, mitochondria become smaller, and mitochondrial cristae decrease or disappear. Through these typical characteristics, different cell death methods can be better distinguished (Parzych & Klionsky, 2014).

7.3.2 | Procedure

Control and treated cells were collected and fixed with electron microscope fixative at 4°C for 4 h. Centrifuge and wash with 0.1 M phosphate buffer (pH 7.4). Fix cells in the dark with 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) at 20°C for 2 h. After fixation, wash cells three times for 10 min each. Dehydrate cells sequentially in 30%–50%–70%–80%–95%–100%–100% ethanol for 20 min each, followed by two changes of 100% acetone for 15 min each. Infiltrate samples overnight in acetone and embedding resin, then embed in embedding molds and polymerize overnight at 37°C followed by 48 h at 60°C. Section samples using an ultramicrotome. Stain copper grids with 2% uranyl acetate were saturated in ethanol in the dark for 8 min, washed and stained with 2.6% lead citrate solution for 8 min, and then washed and dried. Observe the cells under a transmission electron microscope and capture images (Deng et al., 2020).

8 | DETERMINATION OF CELL BEHAVIOR AND FUNCTION

8.1 | Scratch assay

8.1.1 | Principle

The scratch assay involves creating a "scratch" on a monolayer of cells and observing the process of cell migration to fill the scratch, thereby assessing cell migration capability (Martinotti & Ranzato, 2020).

8.1.2 | Procedure

Seed the cells into a 6-well plate and allow them to grow to a confluent monolayer (80%–90% confluence). Create a straight-line wound in the middle of each well using a sterile 20-μL pipette tip. Gently wash the wells twice with PBS to remove cell debris. Capture initial images of the scratch under a microscope to record the width and morphology of the wound. Continue to culture the cells in medium and at appropriate time intervals (12, 24, and 36 h), capture images of the scratch area to observe cell migration toward the scratch. Use Image J software to measure the width or area of the scratch from the captured images (Pijuan et al., 2019).

8.2 | Cell migration and invasion assay

8.2.1 | Principle

The transwell assay is a widely used in vitro method to study cell migration and invasion capabilities. It utilizes transwell chambers with porous membranes where cells migrate through the pores toward a chemoattractant in the lower chamber. The invasion assay includes coating the transwell chamber membrane with a substance that mimics the extracellular matrix (Matrigel), which cells must invade through to reach the lower chamber, thereby simulating the process of cell invasion (Erdogan & Turkekul, 2020; Glei et al., 2016; Justus et al., 2023).

8.2.2 | Experimental procedure

For cell migration: Place transwell chambers with 8 μm pore size inserts into a 24-well plate. Add 100 μL of cell suspension (typically in serum-free medium) to the upper chamber of each transwell insert. Add 500 μL of complete medium (containing 10% fetal bovine serum) to the lower chamber. Incubate in a cell culture incubator for 24 h to allow cell migration through the membrane pores to the lower chamber. Carefully remove the transwell inserts, discard the medium, wash with PBS twice, fix with methanol, wash again, and stain with 0.1% crystal violet in the dark for 20 min. Place the inserts on glass slides, observe under an optical microscope, randomly select 5 fields of view, capture images, and count migrated cells (Hulkower & Herber, 2011; Liu et al., 2018).

For cell invasion: Coat the upper chamber of transwell inserts with 100 μL of diluted matrix gel (matrix gelmedium = 1:3), and allow it to solidify at 37°C. Proceed with the remaining steps similar to the cell migration assay. After 24 h of incubation at 37°C, wash with PBS, fix, stain, and invert the inserts onto glass slides, observe under a microscope, capture images, and count the invaded cells (Wang, Xie, et al., 2022).

8.3 | Colony formation assay

8.3.1 | Principle

Colony formation assay is an experimental method used to assess the proliferative and colony-forming abilities of individual cells. In this assay, single cells or low-density cell suspensions are evenly dispersed onto the surface of a culture dish to proliferate and form

colonies. The number of colonies formed reflects the clonogenic and proliferative capabilities of the cells. This assay is commonly employed to study cell clonogenicity, tumor cell proliferation, and the effects of drugs on cell proliferation (Kabakov & Gabai, 2018).

8.3.2 | Procedure

Seed a suitable number of cells (500–1000 cells) in suspension evenly into a 6-well culture plate. Ensure even distribution of cells without clumping. Incubate the plate in a cell culture incubator for 2 weeks or until visible cell colonies form. Carefully aspirate the medium, wash the cells with PBS, and then fix the cells with an appropriate fixative (such as methanol or 70% ethanol) for 10 min. Wash the cells with PBS. Then, stain with 0.5% crystal violet staining solution for 30 min, ensuring all colonies are adequately stained. Rinse off the crystal violet stain and allow the culture plate to air dry. Use a microscope to observe the colonies in the culture plate, count the number of colonies formed, and calculate the colony forming efficiency (Zhang, Zheng, et al., 2023).

$$\text{Colony Forming Efficiency} = (\text{Number of colonies formed} / \text{Number of cells seeded}) \times 100\%$$

9 | ANTICANCER MOLECULAR MECHANISM DETERMINATION

Cell death includes apoptosis, pyroptosis, autophagy, ferroptosis, etc. Markers can be detected by means of quantitative PCR (q-PCR), western blotting, and immunohistochemistry to determine the cell death mode. Markers of apoptosis are Caspase-3 and PARP. Markers of pyroptosis are Caspase-1, Caspase-4, and Caspase-5. Markers of autophagy are Atg5, Atg7, Beclin I, LC3, and P62. Markers of ferroptosis are SLC7A1, GPX4, and ACSL4.

9.1 | Quantitative PCR

9.1.1 | Principle

Quantitative PCR (q-PCR) is a method used to quantitatively detect DNA molecules. Its principle involves real-time monitoring of fluorescence signal intensity during PCR amplification using fluorescent dyes or probes, allowing for the quantitative determination of the initial amount of target DNA (Singh & Roy-Chowdhuri, 2016).

9.1.2 | Experimental procedure

Mix cells with Trizol to ensure thorough cell lysis. Centrifuge at 10,000×g for 5 min and discard the precipitate. Add chloroform at a ratio of 1:5 and vigorously shake the mixture. After standing, centrifuge for 20 min (10,000×g, 4°C) and transfer the aqueous phase to another centrifuge tube. After adding an equal volume of isopropanol, centrifuge for 10 min (10,000×g, 4°C) and precipitate the RNA to the bottom of the tube. Add 75% ethanol, vortex, and centrifuge again. The ethanol solution was discarded and the RNA precipitate was placed in a sterile ventilated kitchen for drying. Diethypyrocarbonate water was added to dissolve the RNA precipitate. Ensure RNA purity as well as RNA concentration by determining the OD_{260}/OD_{280} ratio using a microspectrophotometer, purity should be between 1.8 and 2.2. After reverse transcription of total RNA samples using a cDNA synthesis kit, q-PCR was performed using a qPCR kit. The relative expression level of the target gene was determined using the $2^{-\Delta\Delta}$Ct method, with β-actin as the reference.

9.2 | Western Blotting

9.2.1 | Principle

Western blotting is a method used to detect the presence and relative quantity of specific proteins in complex mixtures. Proteins extracted from samples are separated by electrophoresis, transferred from the gel to a membrane, and nonspecific binding sites on the membrane are blocked using a blocking reagent. Finally, protein expression levels are analyzed using immunodetection (Sule et al., 2023).

9.2.2 | Sample preparation

Collect cells for protein extraction and wash with PBS three times. Add 100–200 μL cell lysis buffer (containing 1% phenyl methane sulfonyl fluoride) per 1×10^6 cells/mL, lyse on ice for 20–30 min, and shake every 5 min. Centrifuge at 10,000×g for 20 min and collect the supernatant. Determine protein concentration using the bicinchoninic acid assay and record. Mix the supernatant with loading buffer (5×) at a 1/4 ratio, boil for 10 min, cool on ice, and store at −80°C (Pillai-Kastoori et al., 2020).

9.2.3 | WB experimental

Prepare separating gel in a clean glass plate with appropriate concentration (Table 4), quickly overlay with ethanol, wait 20–30 min for gel to solidify. Remove ethanol, add concentrated gel, insert comb of appropriate width and number of wells, wait for 30 min for gel to solidify, then remove comb to obtain gel plate. Place gel plate into electrophoresis chamber, add electrophoresis buffer, load 2 μL standard protein marker and 20 μg sample protein, perform electrophoresis at 80 V initially, then increase to 120 V as proteins enter the separating gel, until electrophoresis is complete. After electrophoresis, remove the separating gel, assemble the gel sandwich (sponge—filter paper—separating gel—polyvinylidene fluoride (PVDF) membrane—filter paper—sponge), ensuring no air bubbles are formed between the

TABLE 4 Formulation of separating gel and stacking gel.

	20 ml			
Separating gel	**8%**	**10%**	**12%**	**15%**
H_2O	9.3	7.9	6.6	4.6
30% acrylamide	5.3	6.7	8.0	10.0
1.5 M Tris (pH 8.8)	5.0	5.0	5.0	5.0
10% SDS	0.2	0.2	0.2	0.2
10% $(NH4)_2S_2O_8$	0.2	0.2	0.2	0.2
TEMED	0.012	0.016	0.020	0.024
	5%			
Concentrated gel	**4 ml**	**6 ml**	**8 ml**	**10 ml**
H_2O	2.70	4.10	5.40	6.80
30% acrylamide	0.67	1.00	1.34	1.70
1.0 M Tris (pH 6.8)	0.50	0.75	1.00	1.25
10% SDS	0.04	0.06	0.08	0.10
10% $(NH4)_2S_2O_8$	0.04	0.06	0.08	0.10
TEMED	0.004	0.006	0.008	0.01

layers. Place in the transfer apparatus, add transfer buffer, transfer at 250 mA constant current for 40–60 min. Remove PVDF membrane and block in 5% skim milk on shaker at room temperature for 2 h. Wash the membrane with phosphate buffered saline with tween-20 (PBST), incubate with primary antibody solution diluted per manufacturer's instructions overnight at 4°C. After overnight incubation, wash membrane with PBST three times, 10 min each, and incubate with secondary antibody solution at 37°C on shaker for 1 h. Wash the membrane again with PBST three times and then proceed to develop and capture images (Chehade et al., 2021; Kurien & Scofield, 2015).

9.3 | Immunofluorescence

9.3.1 | Principle

IF is a method used to detect specific proteins in cells or tissues. It involves using antibodies that bind to the target protein, followed by detection using fluorescent-labeled secondary antibodies or fluorescent dyes to observe and localize the target protein (Im et al., 2019).

9.3.2 | Experimental procedure

Culture cells in six-well plates for 24 h, treat with drugs, wash cells with PBS, and fix with 4% paraformaldehyde. Permeabilize cells with 0.1% Triton X-100 for 10 min, block nonspecific binding sites with 0.1% bovine serum albumin. Incubate cells with primary antibody (1:200) in the dark overnight at 4°C. Wash cells with PBS three times, 5 min each. Incubate with secondary antibody and DAPI for 1 h and 30 min, respectively, at room temperature. Transfer cells onto cover slips, gently press to remove air bubbles. Use a fluorescent microscope to observe samples, capture and record images for analysis (Hussaini et al., 2023).

10 | CONCLUSION

This guide provides a detailed overview of various assays used in cancer research using cancer cell models to study the anticancer properties of drugs or food substances. It covers the selection of cell lines, cell culture techniques, and the determination of physicochemical and molecular mechanisms of anticancer effects. Choosing appropriate cell lines, optimizing experimental conditions, and ensuring the normal growth of cancer cells are crucial. Assessments of cell viability, apoptosis, ROS generation, migration, and invasion provide comprehensive means to evaluate the efficacy of anticancer agents. Each method has unique advantages and applications, and their combined use allows for a more comprehensive understanding of the anticancer effects of drugs or food substances. It is recommended to use more than three methods to verify the accuracy of experimental results. Furthermore, q-PCR, western blotting, and IF reveal the molecular mechanisms underlying the anticancer effects of drugs or food substances, providing robust support for research on anticancer agents or foods.

AUTHOR CONTRIBUTION

Fei-Fei Ma: Conceptualization; Investigation; Writing - original draft; Methodology; Writing - review & editing.

CONFLICT OF INTEREST STATEMENT

There is no declaim.

DATA AVAILABILITY STATEMENT

Research data are not shared.

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How to cite this article: Ma, F.-F. (2024). Guideline for anticancer assays in cells. *Future Postharvest and Food*, *1*(3), 348–359. https://doi.org/10.1002/fpf2.12039